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INITIATION AND GROWTH OF *LEUCOSPERMUM CORDIFOLIUM* CALLUS

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ABSTRACT

Callus was initiated from the cotyledons and hypocotyls of *Leucospermum cordifolium* seedlings. A comparative study of four media with respect to their ability to support the growth of this callus revealed that Miller's (1965) medium was best suited for this purpose.

UITTREKSEL

INISIASIE EN GROEI VAN *LEUCOSPERMUM CORDIFOLIUM* KALLUS

Kallus is van die saadlobbe en hipokotiele van *Leucospermum cordifolium* saailinge geïnisieer. 'n Vergelykende studie van vier media ten opsigte van hulle vermoë om die groei van dié kallus te onderhou het getoon dat die medium van Miller (1965) die beste hiertoe instaat is.

INTRODUCTION

Propagation by tissue culture is an ideal and widely used method for the vegetative multiplication of particular clones of plants which are difficult to propagate by standard horticultural techniques. Very small pieces of plant tissue e.g. seeds, embryos, stems, midribs or inflorescences have been cultured to establish callus which can subsequently be used for micro-propagation of the plant concerned (Hartmann and Kester, 1975).

A number of species of the South African Proteaceae, in particular *Orothamnus zeyheri*, are extremely rare. On two occasions it has been thought to be extinct. At present, very few plants of this monotypic genus are found in the wild. Attempts to obtain seed by hand pollination of flowers proved successful. However, germination of this seed has been poor and sporadic (Van der Merwe, 1974). Because of the success of tissue culture techniques in propagation of other horticulturally desirable plants (Morel, 1964; Romberger *et al.*, 1970; Ziv *et al.*, 1970; Ben-Jaacov and Langhans, 1970; Pierik *et al.*, 1974) these techniques appear to be ideally suited to overcome the propagation difficulties of this rare plant. In view of the limited material available initial experiments were conducted on a closely related plant, *Leucospermum cordifolium*. This was done with the object of establishing means of initiating callus and the selection of a nutrient medium best suited to support its continuous growth.

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EXPERIMENTS AND DISCUSSION

Leucospermum cordifolium (Salisb. ex Knight) Fourcade seed, obtained from the Forestry Department, Pretoria, was used as experimental material. After scraping off the thin, brittle, outer pericarp seeds were surface sterilized for 30 minutes in a saturated calcium hypochlorite solution. Following this they were immersed in 95% ethanol for 10 minutes and then thoroughly rinsed with sterile distilled water. Previous experiments have shown that germination of this species is retarded by the presence of the hard, woody, inner pericarp as it restricts oxygen uptake by the embryo (Van Staden and Brown, 1973). As a result the woody pericarp was removed from all surface sterilized seed under aseptic conditions. The excised embryos were transferred to 100 ml flasks containing 50 ml of Hoagland's solution solidified with 1% agar. The flasks were kept in a germinator and maintained at a daily temperature regime of 10°C for 16 hours followed by 20°C for 8 hours. Sterilization of the embryos was 64% effective. Sterile embryos started germinating after 15 days. After 30 days 69% of these embryos had germinated and developed into seedlings. When six weeks old the cotyledons and hypocotyls were excised from the seedlings and the explants cultured on Miller's (1965) soybean medium. In addition to the basic salts and vitamins sucrose (3%), NAA (2 mg/1), coconut milk (10%) and Difco Bacto-agar (1%) were incorporated into the medium. The pH of the medium was adjusted to 5,8 and 40 ml dispensed into 100 ml flasks which were then autoclaved. Cultures containing explants were maintained under continuous light from white fluorescent tubes at 26 ± 1°C. Both cotyledonary and hypocotyl explants showed signs of callus initiation after eight weeks of culturing. This callus grew slowly and required sub-culturing approximately every six weeks.

Callus of cotyledonary and hypocotyl origin was very different in appearance: that from the cotyledons was hard and green, while in contrast callus from hypocotyls was white, soft and friable in appearance (Fig. 1A).

Although the original choice of medium proved most fortunate in that it was capable of initiating and supporting the growth of *Leucospermum* callus, it was nevertheless essential to establish whether other media could not accelerate the rate of callus growth. As insufficient hypocotyl callus was available, subsequent experiments were carried out with cotyledonary callus only. All experiments were repeated twice and treatments replicated ten times. The constituents of the media tested for their ability to support the growth of *Leucospermum* callus are given in Table 1. Sucrose (3%), Difco Bacto-agar (1%) and NAA (2 mg/1) were incorporated into all media. The efficacy of coconut milk (10%) was compared with the naturally-occurring cytokinin zeatin (10 μg/1) as preliminary experiments indicated that kinetin was less effective in maintaining callus growth. In addition it is now known that coconut milk contains zeatin

(Van Staden and Drewes, 1975) and that this cytokinin is present in proteaceous species (Brown and Van Staden, 1973; Van Staden and Davey, 1976). Before autoclaving, the pH of each medium was adjusted to 5,8 and 40 ml dispensed

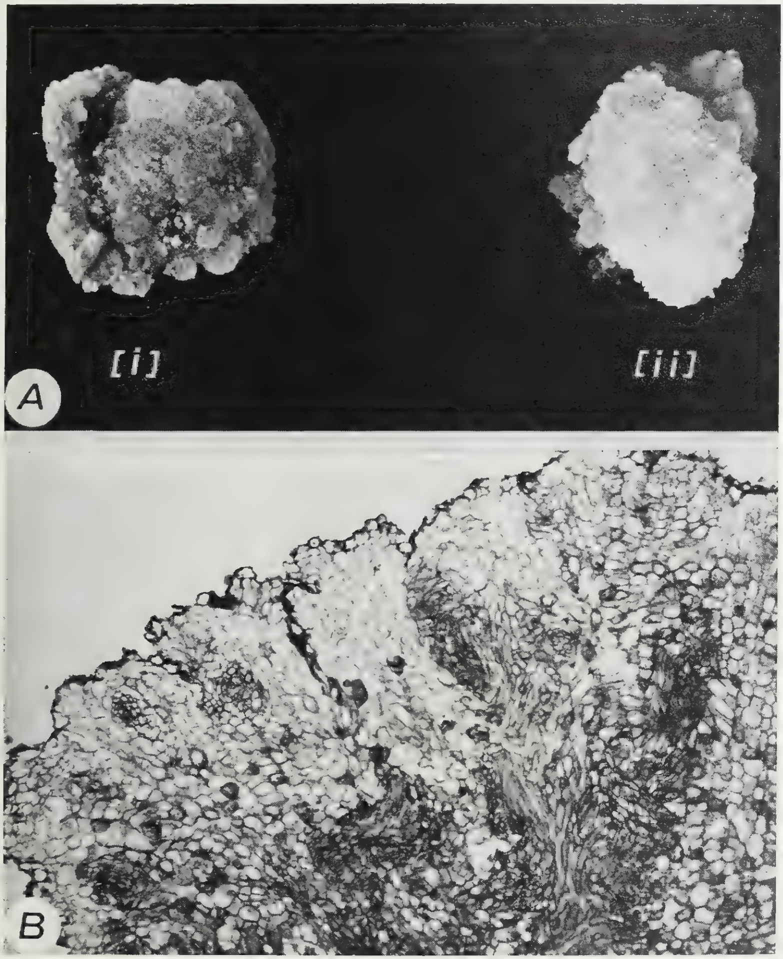

Fig. 1A.
Appearance of *Leucospermum* callus of (i) cotyledonary and (ii) hypocotyledonary origin.
Fig. 1B.
Islets of meristematic cells in callus grown on Miller's (1965) medium in the presence of zeatin.

into 100 ml flasks. One piece of callus (± 30 mg) was transferred to each flask and allowed to grow for 60 days under the conditions described earlier. At the end of this period the callus was weighed. From the data presented in Fig. 2 it is obvious that Miller's medium was most beneficial for the growth of *Leucospermum* callus. No simple explanation can be given for these results. A comparison of the composition of the media investigated (Tables 1 and 2) indicates that the vitamin concentration, especially thiamine, nicotinic acid and pyridoxine of Miller's medium was much lower than that used by Murashige and Tucker (1969) and Schenk and Hildebrandt (1972). The micro-nutrient composition of most media showed only slight variation. In the case of Heller's medium however, the trace element molybdenum was omitted. This together with the fact that nitrogen was supplied as nitrate only may account for the poor growth of the callus on this medium, especially as this essential element is required for nitrate reduction (Steward and Durzan, 1965). A further contributing factor may be the fact that except for potassium the concentrations of essential macronutrient elements supplied are extremely low (Table 2). The major differences recorded (Fig. 2) between the media of Murashige and Tucker (1969), Schenk and

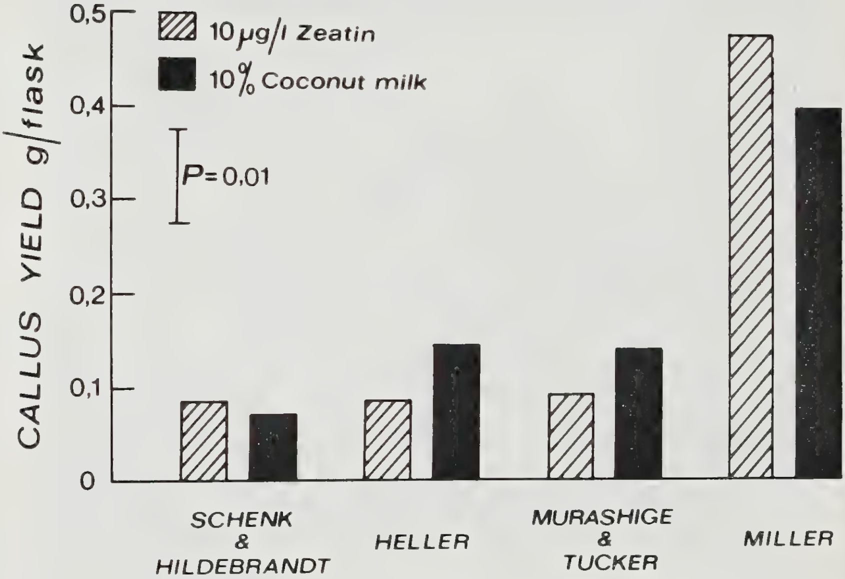

FIG. 2.
The effect of different nutrient media on the growth of *Leucospermum* callus.

TABLE 1.

Constituents of media tested for their ability to maintain the growth of *Leucospermum* callus.

Constituent	Weight of constituents in final medium (mg/liter)			
	Heller (1953)	Murashige and Tucker (1969)	Miller (1965)	Schenk and Hildebrandt (1972)
Macro-nutrients				
NH_4NO_3	—	1 650	1 000	—
$NH_4H_2PO_4$	—	—	—	300
KNO_3	—	1 900	1 000	2 500
KCl	750	—	65	—
KH_2PO_4	—	170	300	—
$NaNO_3$	600	—	—	—
$NaH_2PO_4 \cdot H_2O$	125	—	—	—
$CaCl_2$	75	—	—	—
$CaCl_2 \cdot 2H_2O$	—	440	—	200
$Ca(NO_3)_2 \cdot 4H_2O$	—	—	500	—
$MgSO_4 \cdot 7H_2O$	250	370	71,5	400
Micro-nutrients				
$FeCl_3 \cdot 6H_2O$	1,0	—	—	—
NaFeEDTA	—	40,0	13,2	40,0
H_3BO_3	1,0	6,2	1,6	5,0
$ZnSO_4 \cdot 7H_2O$	1,0	8,6	3,8	1,0
$MnSO_4 \cdot 4H_2O$	0,01	22,3	14,0	10,0
$CuSO_4 \cdot 5H_2O$	0,03	0,025	—	0,2
$Cu(NO_3)_2 \cdot 3H_2O$	—	—	0,35	—
$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	—	—	0,1	—
$Na_2MoO_4 \cdot 2H_2O$	—	0,25	—	0,1
KI	0,01	0,83	—	1,0
$CoCl_2 \cdot 6H_2O$	—	0,025	—	0,1
$AlCl_3$	0,03	—	—	—
$NiCl_2 \cdot 6H_2O$	0,03	—	—	—
Vitamins				
Myo-inositol	Vitamins of Miller (1965) added	100	100	1 000
Thiamine·HCl		10	0,1	5
Nicotinic acid		5	0,5	5
Pyridoxine·HCl		10	0,1	5
Glycine		2	—	—

Hildebrandt (1972) and Miller (1965) can probably be attributed to differences in macro-nutrient composition and/or the ratio of ammonia nitrogen: nitrate nitrogen incorporated into them. It has previously been shown that a balanced supply of nitrate and ammonia salts is beneficial to the growth of certain types of callus (Shantz and Steward, 1959). In Table 2 it can be seen that the media of Murashige and Tucker (1969) and Miller (1965) have roughly similar ratios of ammonia: nitrate. In contrast that of Schenk and Hildebrandt (1972) contains a very low concentration of ammonia nitrogen. This low concentration may possibly explain its detrimental effect on the callus growth. The major difference, apart from their vitamin complement, between Murashige and Tucker's and Miller's media is that the latter contained substantially lower levels of macronutrient elements (Table 2), the exception being phosphorus which was present in a higher concentration.

The callus pieces grown on Miller's medium in the presence of zeatin not only grew well, but on their lower surface contained small dome-shaped outgrowths. This material was fixed in formalin-acetic acid-alcohol, dehydrated and embedded in the usual manner for histological investigation.

The callus proved to be composed of parenchymatous cells. In places islets of meristematic tissue were observed in which the cells contained a dense cytoplasm. Within these islets vascular tissue, mainly phloem, had differentiated (Fig. 1B). In view of the fact that in other plants these islets develop into adventitious buds or "embryoids" (Bajaj and Mäder, 1974), it seems reasonable to assume that *Leucospermum* callus may have the same ability. Whether this will happen depends on the balance of hormones supplied to this tissue. This problem is currently under investigation.

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TABLE 2.
Macro-nutrient element composition of media compared for their ability to support the growth of *Leucospermum* callus. (*Concentration of elements in final medium. mg/l*)

Element	Heller (1953)	Murashige and Tucker (1969)	Miller (1965)	Schenk and Hildebrandt (1972)
N	98,8	840,0	547,8	382,5
P	24,8	39,0	68,3	80,8
K	392,3	782,0	120,0	964,3
Ca	27,0	121,0	84,7	54,4
Mg	24,8	36,8	7,0	38,9
S	32,4	52,3	9,3	51,9
Ratio of $NH_4^+:NO_3^-$	Nitrogen as NO_3^- only	1:1,9	1:2,1	1:9,5

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